DNA Polymerase zeta is a major determinant of resistance to platinum-based chemotherapeutic agents

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Running title: REV3 deficiency sensitizes cells to platinum-based drugs

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Abbreviations: TLS, translesion DNA synthesis; Polη, DNA polymerase eta; Polζ, DNA polymerase zeta; Polθ, DNA polymerase iota; ICL, interstrand DNA crosslink; DSBs, DNA double-stranded breaks; RPA, replication protein A; ATR, ataxia telangiectasia and Rad3 related; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis).
Abstract

Oxaliplatin, satraplatin, and picoplatin are cisplatin analogs that interact with DNA forming intrastrand and interstrand DNA crosslinks (ICLs). Replicative bypass of cisplatin DNA adducts requires the cooperative actions of at least three translesion DNA synthesis (TLS) polymerases: eta (Polη), REV1, and zeta (Polζ). Because oxaliplatin, satraplatin, and picoplatin contain bulker chemical groups attached to the platinum core as compared to cisplatin, we hypothesized that these chemical additions may impede replicative bypass by TLS polymerases and reduce tolerance to platinum-containing adducts. We examined multiple responses of cancer cells to oxaliplatin, satraplatin, or picoplatin treatment under conditions where expression of a TLS polymerase was limited. Our studies revealed that although Polη contributes to the tolerance of cisplatin adducts, it plays a lesser role in promoting replication through oxaliplatin, satraplatin, and picoplatin adducts. REV1 and Polζ were necessary for tolerance to all four platinum analogs and prevention of hyper-activation of the DNA damage response following treatment. In addition, REV1 and Polζ were important for the resolution of DNA double-stranded breaks (DSBs) created during replication-associated repair of platinum-containing ICLs. Consistent with ICLs being the predominant cytotoxic lesion, depletion of REV1 or Polζ rendered two different model cell systems extremely sensitive to all four drugs, whereas Polη depletion had little effect. Together, our data suggest that REV1 and Polζ are critical for promoting resistance to all four clinically relevant platinum-based drugs by promoting both translesion DNA synthesis and DNA repair.
Introduction

Cisplatin is widely used for the treatment of a broad range of malignant diseases, including testicular, ovarian, lung, and bladder cancer (Kelland, 2007). The antitumor effect of cisplatin is through its ability to covalently interact with guanine residues in DNA resulting in the formation of both intra- and interstrand DNA crosslinks (ICLs). Although cisplatin is an effective anticancer drug in several tumor types, its usefulness can become limited due to severe dose-limiting side effects and acquired resistance (Koberle et al., 2010; Rabik and Dolan, 2007). Since the introduction of cisplatin, additional analogs have been developed with the goal of reducing toxicity, broadening the spectrum of activity, and circumventing acquired resistance (Kelland, 2007). Modifications to the leaving groups or the two amine ligands of cisplatin resulted in the development of carboplatin and oxaliplatin respectively, both currently approved for use in the United States. Carboplatin was developed to lower the toxicity profile of cisplatin by replacing the dichloride leaving groups with 1,1-cyclobutanedicarboxylate. This resulted in a cisplatin-like compound that generates the same DNA adducts, but is more stable and undergoes aquation at a slower rate. Oxaliplatin was designed with a 1,2-diaminocyclohexane (DACH) group in place of the two amine ligands based on the prediction that a bulkier platinum-DNA adduct would interfere with DNA repair and overcome cisplatin resistance.

Continuous efforts to improve efficacy and bioavailability of platinum-based chemotherapeutic agents resulted in the development of two newer analogs that are currently being evaluated in clinical trials. Satraplatin (JM216) was developed to circumvent acquired resistance by replacing one of the amine ligands of cisplatin with a bulkier cyclohexylamine group. Picoplatin (AMD473) contains a pyrimidine ring substituted for one of the amine ligands of cisplatin in order to prevent the platinum center from being inactivated by glutathione. Even
though all these analogs interact with DNA in a similar manner (Figure 1A), it is becoming increasingly clear that the different adducts produced by these compounds are associated with distinct spectrums of activity, and in some cases, an inability of specific proteins to recognize the lesion thus leading to altered cellular responses (Chaney et al., 2005; Lewis et al., 2009; Nehme et al., 1999; Raymond et al., 2002).

The overall cellular response to platinum-DNA intrastrand crosslinks and ICLs involves multiple processes that ultimately determine cell fate. We and others have identified TLS as an important pathway influencing cisplatin-induced cytotoxicity (Albertella et al., 2005; Bassett et al., 2004; Chen et al., 2006; Doles et al., 2010; Hicks et al., 2010; Niedzwiedz et al., 2004; Nojima et al., 2005; Okuda et al., 2005; Simpson and Sale, 2003; Sonoda et al., 2003; Wu et al., 2004). The TLS pathway promotes tolerance to various DNA lesions that block replicative polymerases and is triggered by the monoubiquitination of proliferating cell nuclear antigen (PCNA) by the RAD6 (E2)-RAD18 (E3) complex (Waters et al., 2009). Monoubiquitination of PCNA on Lys164 recruits TLS polymerases through a combination of protein-protein interacting motifs that include PCNA-interacting peptide (PIP) and ubiquitin-binding domains. TLS polymerases possess accommodating active sites and are capable of replicating DNA containing bulky DNA lesions, even when the DNA template is distorted by adducts such as those created by cisplatin (Alt et al., 2007; Bhattacharyya et al., 2011; Washington et al., 2010; Waters et al., 2009).

Multiple TLS polymerases are implicated in the lesion bypass of DNA intrastrand crosslinks, including those generated by cisplatin. Both DNA polymerase eta (Polη) and DNA polymerase zeta (Polζ, composed of catalytic subunit REV3 and accessory subunit REV7) are believed to cooperate together when synthesizing DNA opposite cisplatin adducts and this
activity requires the TLS polymerase REV1 (Hicks et al., 2010; Shachar et al., 2009). Current models suggest that REV1 facilitates polymerase switching during TLS through its ability to bind multiple TLS polymerases (Guo et al., 2003; Ohashi et al., 2004; Tissier et al., 2004). Currently, it is unclear whether Polη and/or REV1/Polζ-dependent TLS mediates resistance to the newer platinum-based drugs that based on their structure, could create larger obstructions to DNA replication including TLS. Here we demonstrate that cancer cells lacking REV1 or REV3 are highly sensitive to these agents, show markedly reduced survival, and fail to resolve replication-associated DSBs after treatment with cisplatin, oxaliplatin, satraplatin, or picoplatin. In contrast, we found that Polη appeared to play a relatively small role in promoting resistance to these drugs. Together our findings are consistent with REV1 and Polζ being key factors in promoting resistance to platinum-based chemotherapy and hence support the rationale of TLS inhibition as an adjuvant therapy for treating malignancies that develop chemoresistance.
Materials and Methods

Reagents. Cisplatin (cis-diaminedichloroplatinum(II)), oxaliplatin (1R,2R-diaminocyclohexane oxalatoplatinum(II)) and picoplatin (cis-amminedichloro, 2-methylpyridine, platinum(II)) were purchased from LC laboratories (Woburn, Massachusetts). Satraplatin (bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV)) was purchased from Sequoia Research Products (United Kingdom). Rabbit polyclonal anti-Polη (H-300), anti-53BP1 (H-300), anti-GAPDH (FL-335) and anti-Rad51 (H92) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit polyclonal anti-phospho RPA32 (S4/S8), anti-phospho-Ser345 CHK1, anti-phospho-Ser139 Histone H2AX, and anti-RAD18 were purchased from Bethyl Laboratories (Mongomery, Texas), Cell Signaling Technology (Danvers, Massachusetts), Active Motif (Carlsbad, California) and Proteintech Group (Chicago, Illinois), respectively. The following mouse monoclonal antibodies were used: anti-phospho-Ser 1981 ATM (Rockland Immunochemicals Inc., Gilbertsville, Pennsylvania), anti-GFP (Roche Applied Science, Indianapolis, Indiana), anti-Chk1 (G-4; Santa Cruz Biotechnology), anti-RPA32 (Ab-3, Calbiochem/ EMD Chemicals, Billerica, MA), and anti-Topoisomerase 1 (BD Biosciences, San Diego, California).

Cell lines, siRNA and culture conditions. HeLa cells were obtained from the American tissue culture collection (ATCC) and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The BL2 human Burkitt’s lymphoma cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS (Gueranger et al., 2008). All siRNA duplexes were purchased from Qiagen (Valencia, California) and transfected into HeLa cells using X-tremeGENE Transfection Reagent (Roche Applied Science) as
described (Hicks et al., 2010). The gene sequences used for designing siRNA employed in this study are: siControl (AATTCTCCGAACGTGTCACGT), siREV1 (ATCGGTGGAATCGGTTTGAA), siREV3 (CCCAGTTGATTAATGCACAA), Polη (CAGCCAAATGGCCCATTCGCAA), siRAD18 (ATGGTTGTGGCCCGAGGTAA), and siRAD51 (AAGCTGAAGCTATGTTCGCCA).

Measurement of cell growth and loss in viability. To measure the extent of growth inhibition caused by treatment with platinating agents, Hela cells were seeded 10,000 cells/well in 12-well plates and exposed to drug for one hour. Four days later, cells were harvested and counted using an Accuri C6 flow cytometer. For survival assays, HeLa cells were transfected overnight with each siRNA as described (Hicks et al., 2010). The next day, cells were seeded at known densities (500-2000 cells/well) in 12-well plates, allowed to attach to culture plates overnight, and then cultured with two different doses of cisplatin, oxaliplatin, satraplatin or picoplatin until the untreated well approached confluency (7-10 days). Cellular survival was determined using a crystal violet assay, as described previously (Taniguchi et al., 2002). BL2 lymphoma cells were treated with the different platinum analogs continuously for 48 hours. The cells were then washed with phosphate buffered saline and viability was determined by measuring the percentage of BL2 cells excluding propidium iodide using an Accuri C6 flow cytometer.

Immunofluorescence. For γ-H2AX immunofluorescence, HeLa cells grown on coverslips in 12-well plates were transfected overnight with the different siRNAs and allowed to recover another 24 hours. The transfected cells were treated for one hour with the drug concentration that inhibited cell growth by 35 to 40% (as measured using the growth inhibition assay; 10 μM cisplatin, 32 μM oxaliplatin, 6 μM satraplatin and 64 μM picoplatin). Cells were
fixed with 100% methanol 24 hours later and stained for γ-H2AX as described (Hicks et al., 2010). To analyze the presence of DSBs by immunofluorescence, HeLa cells were transfected with siRNA as above, then treated with 5 μM cisplatin, 15 μM oxaliplatin, 2 μM satraplatin or 25 μM picoplatin for one hour. Cells were fixed with 100% methanol 24 and 48 hours after drug treatment and stained for S1981P-ATM and 53BP1 as described (Hicks et al., 2010). The doses chosen were based on experiments included in Supplemental data, Figures 2 through 5.

**Flow Cytometry.** HeLa cells were transfected overnight with individual siRNAs and allowed 24 hours to recover. Cells were then treated with 10 μM cisplatin, 32 μM oxaliplatin, 6 μM satraplatin and 64 μM picoplatin for 1 hour, washed, harvested 24 hours later, and fixed in 70% methanol. For two parameter flow cytometry, cells were blocked with 5% bovine serum albumin (BSA) plus 1% goat serum and 0.05% Tween-20, and then stained with γ-H2AX monoclonal antibody. Cells were washed, incubated with the goat anti-mouse FITC conjugated secondary antibody (Sigma-Aldrich, St. Louis, Missouri) and then counterstained with propidium iodide in PBS containing RNase A. Cells were acquired on an Accuri C6 flow cytometer (Ann Arbor, Michigan).

**Immunoblotting.** Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer: 10 mM Tris pH 8.0, 2% SDS, 1X protease inhibitor cocktail (Roche Applied Science), 1X phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich). Lysates were sonicated, and then denatured by heating to 95°C for 5 minutes. Equal amounts of protein were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and then probed with the appropriate primary antibodies followed by secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit or mouse antibodies (Thermo Scientific, Rockford, IL). Proteins were visualized using SuperSignal West Pico Chemiluminiscent Substrate (Thermo Scientific).
Statistics. Statistically significant differences were determined using one-way ANOVA with the Tukey post-comparison test using GraphPad PRISM version 5 (GraphPad Software, La Jolla, CA).
Results

RAD18, Polη, REV1 and REV3 promote different degrees of tolerance to cisplatin analogues. We have previously determined that exposing HeLa cells to 10 μM cisplatin for one hour inhibits cell proliferation by approximately 35 to 40%. Following this treatment, we were able to measure significant differences in survival and activation of the DNA damage response pathway in cells depleted of various components of the TLS pathway (RAD18, Polη, REV1 or REV3) (Hicks et al., 2010). Since platinum analogues can form DNA adducts with different efficiencies after entering a cell and could pose different degrees of replication blockade, we first compared the ability of HeLa cells to proliferate after treating cells with oxaliplatin, satraplatin, and picoplatin for one hour in order to identify comparable doses that would lead to 35-40% inhibition of cell growth (Figure 1B). Based on this analysis, we chose to treat siRNA-transfected HeLa cells for one hour with 32 μM oxaliplatin, 6 μM satraplatin, or 64 μM picoplatin and compared responses of cells to 10 μM cisplatin using various endpoints that indirectly measure the stalling of replication forks. We presumed that under these conditions, we are comparing responses of cells to equivalent levels of replication blockade generated by each analog.

Activation of the ATR kinase is a well characterized response to replication blockade (Cimprich and Cortez, 2008). We therefore measured the phosphorylation of ATR substrates (e.g. H2AX and Chk1) as surrogate markers for replication stress as a result of deficient TLS (Cruet-Hennequart et al., 2008; Hicks et al., 2010). We previously determined that HeLa cells treated with cisplatin exhibit intense hyperphosphorylation of H2AX on serine 139 (γ-H2AX) when cells are depleted of REV1, REV3, RAD18, or Polη (Hicks et al., 2010). This exaggerated
response indicates that each protein is necessary for efficient bypass of replication-blocking cisplatin adducts and the avoidance of ATR activation in response to replication fork stalling. We tested whether RAD18, REV1, REV3, or Polη deficiency (validated in Figure 2B, C, and D) leads to replication stalling and activation of ATR following treatment with the different platinum analogs. As expected, HeLa cells deficient in REV1, REV3, RAD18 or Polη expression exhibited robust γ-H2AX staining in comparison to control siRNA transfected cells following exposure to cisplatin (Figure 2A). In contrast, oxaliplatin did not appear to induce the same extent of H2AX phosphorylation in TLS-deficient cells, the exception being siREV3 transfected cells. This experiment also revealed that depletion of Polη was not associated with an exaggerated γ-H2AX response following treatment with oxaliplatin, picoplatin, or satraplatin.

We extended these observations by comparing both time-dependent and dose-dependent induction of H2AX phosphorylation in control and REV3-specific siRNA transfected cells (Supplementary data, Figure S1). The results show that Polζ deficiency resulted in prolonged and extensive H2AX phosphorylation following short term treatment with each cisplatin analog.

To better differentiate intensities of γ-H2AX staining observed by immunofluorescence among the different treatment groups in Figure 2, we analyzed cells treated under identical conditions and stained for γ-H2AX and DNA content by flow cytometry (Figure 3A and B). Differences in both the level of γ-H2AX staining and changes in cell cycle distribution following treatment with the different platinum analogs were revealed more clearly. Notably, only cisplatin appears to induce an exaggerated γ-H2AX response in HeLa cells when Polη is depleted (Figure 3A). When these cells are treated with oxaliplatin, satraplatin or picoplatin, there are relatively small increases in γ-H2AX positive cells (Figure 3A and B). In contrast, the greatest altered cell cycle profiles and intensities of γ-H2AX staining were observed in cells
depleted of REV3, the catalytic subunit of Polζ, regardless of the platinum analog used for treatment (Figure 3A and B). Consistent with REV1 and REV3 cooperating to perform lesion bypass, cells depleted of REV1 exhibited similar cell cycle profiles and cell cycle patterns of γ-H2AX staining (mainly cells residing in S and G2/M) as REV3-depleted cells, the exception being oxaliplatin. REV3-depleted cells showed the greatest shift in γ-H2AX staining following treatment with each of the four platinum analogs.

We and others have previously found that depleting cells of TLS polymerases (Polη or Polζ), as well as abrogating the monoubiquitination of PCNA by RAD18, leads to enhanced ATR-dependent phosphorylation of the Chk1 protein kinase after UV irradiation or cisplatin treatment (Bomgardner et al., 2006; Cruet-Hennequart et al., 2008; Hicks et al., 2010). We therefore compared the response of TLS-deficient HeLa cells following treatment with oxaliplatin, picoplatin and satraplatin as in Figure 2 and 3. Consistent with the degree of γ-H2AX formation and cell cycle arrests in S and G2/M following cisplatin treatment, depletion REV1, REV3, RAD18 or Polη resulted in enhanced phosphorylation of Chk1 on S345 by the ATR kinase (Figure 4A and Supplemental data, Figure S2). However, the extent of Chk1 phosphorylation observed Polη-depleted cells was notably less following treatment with oxaliplatin, picoplatin or satraplatin when compared to REV3-depleted cells. Similar results were observed when phosphorylation of replication factor A (RPA32) on Serines 4 and 8, another marker for replication stress, was analyzed. (Figure 4B and Supplemental data, Figure S2). Again, cells depleted of Polη displayed significantly less phosphorylated RPA as compared to REV3-depleted cells following drug treatment, especially in response to satraplatin. It is also apparent from these analyses that RAD18 is important for most lesion bypass events induced by
oxaliplatin, picoplatin or satraplatin since cells depleted of RAD18 exhibited enhanced phosphorylation of Chk1 and RPA.

**REV1 and Polζ promote resistance to multiple platinating agents.** We next examined the roles of REV1, Polζ and Polη in preventing drug-induced cytotoxicity using two different model systems. REV1, REV3, or Polη-depleted HeLa cells were cultured in the presence of two different concentrations of cisplatin, oxaliplatin, satraplatin, or picoplatin for approximately 8 days and the relative surviving fraction was calculated based on crystal violet staining (Taniguchi et al., 2002). We observed that REV1 and REV3 siRNA-transfected HeLa cells exhibited the greatest sensitivities to cisplatin-, satraplatin-, oxaliplatin-, and picoplatin-induced loss in cell survival, particularly at the lower drug concentrations (Figure 5A). Polη depletion did not appear to sensitize HeLa cells to either platinating agent, at least under the conditions employed here. Consistent with the homologous recombination pathway playing an essential role in repairing interstrand DNA crosslinks (ICLs), depletion of RAD51 caused HeLa cells to be hypersensitive to all four platinating agents, thus validating our experimental approach (Deans and West, 2011). To confirm the predominant role of REV3 in protecting cells from platinum adduct-induced cytotoxicity, we examined the sensitivity of human BL2 lymphoma cells and several different BL2 lines devoid of the REV3, Polη, or Polτ genes to each platinum analog (Gueranger et al., 2008). Consistent with the results observed in REV3-depleted HeLa cells, two different BL2 clones lacking REV3 were significantly more sensitive to each drug (Figure 5B). These results confirm that Polη and Polτ (a member of the Y-family TLS polymerases) play relatively minor roles in promoting resistance to platinum adducts when compared to Polζ.

**REV1 and REV3 depletion leads to defective interstrand crosslink repair.** The extreme sensitivity of REV3 deficient cells to cisplatin has been linked to defective repair of
ICLs (Hicks et al., 2010; Niedzwiedz et al., 2004; Raschle et al., 2008). DNA double-stranded breaks (DSBs) can be detected after cells are exposed to ICL-inducing agents such as mitomycin C or cisplatin, and represent an intermediate step during ICL repair after a replication fork encounters the lesion (Hanada et al., 2006; Hicks et al., 2010; Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Rothfuss and Grompe, 2004). Cleavage on either side of the ICL by the coordinated actions of 5′ and the 3′-flap endonucleases is thought to generate replication-associated DSBs which are subsequently resolved by homologous recombination repair coordinated by the Fanconi anemia complex of proteins (Deans and West, 2011). REV1 and REV3 are believed to promote ICL repair by inserting nucleotides opposite the unhooked ICL, a step that is essential for preparing the damaged DNA template for homologous recombination repair. The appearance and resolution of ICL-induced DSBs can be followed using immunofluorescence microscopy after staining cells with antibodies which specifically detect proteins that associate with chromatin surrounding DSBs forming visible foci. Localization of activated ATM protein kinase (specifically detected as the S1981P-modified form) and 53BP1 to DSBs are both well characterized surrogate markers of DSBs (Bakkenist and Kastan, 2003; Schultz et al., 2000). We therefore examined cisplatin analog-induced colocalization of these two proteins as a sensitive means to follow the appearance and resolution of DSBs created during ICL repair.

HeLa cells were transfected with individual siRNAs, allowed to recover, and then treated relatively low doses of drug: 5 μM cisplatin, 15 μM oxaliplatin, 2 μM satraplatin, or 25 μM picoplatin for 1 hour. Cells were fixed 24 or 48 hours after drug exposure and stained for activated ATM and 53BP1 to follow the induction and resolution of replication-associated DSBs. Lower doses were chosen for this study based on a preliminary experiment assessing the
appearance and resolution of DSBs following treatment of control or REV3 knockdown cells exposed to three different doses of platinating agent (Supplemental data Figures S3 through 5). Following drug treatment, we typically observed the accumulation of DSBs by 24 hours such that approximately 30-50% of cells displayed ten or more colocalized foci marked by S1981P-ATM and 53BP1 (Figure 6A). The percentage of cells displaying 10 or more foci was notably higher in Polη, REV3 or RAD51-deficient cells. By 48 hours, the majority of siControl-transfected cells displayed less than 10 S1981-ATM and 53BP1 colocalized foci indicating that most DSBs have been resolved. In contrast, Rad51-depleted HeLa cells, which are defective in homologous recombination repair (Sharma et al., 2012), failed to resolve DSBs within the 48 hour time period as expected, thus validating our experimental approach. Similar to control cells, Polη-depleted HeLa cells resolved the majority of DSBs within a 48 period after treatment with cisplatin, satraplatin or picoplatin. However, we observed significant differences in the percentage of cells displaying DSBs induced by oxaliplatin in Polη-depleted cells (Figure 6A).

The percentage of drug treated REV3-depleted HeLa cells displaying S1981P-ATM and 53BP1 colocalized foci did not decrease at the 48 hour time point, similar to the RAD51-depleted cells (Figures 6A). Greater than 50% of REV3-depleted cells failed to resolve foci marking DSBs induced by all four drugs within the 48 hour period indicating that DSB repair in these cells was impacted to a large degree (see also supplemental data, Figures S 3 through S5). Since the percentage of drug-treated cells exhibiting DSBs varied at the 24 hour time point, we analyzed the difference in percentage of cells displaying ≥ 10 colocalized foci between 24 and 48 hours in order to examine the impact of DNA polymerase deficiency on DSB resolution during this time period (Figure 6B). This analysis clearly shows that Polη-deficient cells were capable
of resolving DSBs, whereas the percentage of Polζ-depleted cells displaying ≥ 10 foci increased rather than decreased.

**Discussion**

In the present study, we examined the roles of REV1, Polζ, and Polη in protecting cells from the antiproliferative effects of cisplatin in comparison with three cisplatin analogs that produce bulkier adducts on DNA. Previous studies have focused on Polη and its ability to replicate cisplatin and oxaliplatin adducts in template DNA and promote resistance to these agents (Alt et al., 2007; Bassett et al., 2004; Cruet-Hennequart et al., 2008; Vaisman et al., 2000). Our data agree with the concept that Polη is necessary for replicative bypass through cisplatin adducts. However, our data also indicate that Polη does not appear to play a large role in the bypass of oxaliplatin, satraplatin, and picoplatin adducts, at least in comparison to REV1 and Polζ in the context of two different cancer cell lines. It is important to note here that DNA polymerase kappa performs error free bypass of benzo[a]pyrene adducts and error prone bypass of cisplatin adducts in the absence of Polη (Bi et al., 2006; Shachar et al., 2009; Ziv et al., 2009). It will be important to test whether Polκ can fulfill Polη’s role in bypassing bulkier platinum containing adducts during DNA replication with respect to drug-induced mutagenesis and the promotion of cell survival. Regardless, both lesion bypass events initiated by Polη or Polκ require Polζ as the universal extender beyond nucleotides inserted opposite DNA adducts during TLS, likely in collaboration with REV1. Overall, our data point to REV1 and Polζ as being essential for lesion bypass and tolerance of many platinating agents that are used clinically today.

The importance of REV1 and Polζ in facilitating replicative bypass of platinum DNA adducts has important clinical implications. It is well accepted that most mutations induced by DNA damaging agents result from error prone TLS that is attributed to the activities of REV1
and Polζ (Waters et al., 2009). The emergence of drug resistance to cisplatin and cyclophosphamide has recently been linked to the activities of REV3 and REV1 in murine models of B-cell lymphoma and lung adenocarcinoma (Doles et al., 2010; Xie et al., 2010). Rendering tumor cells REV1 or REV3-deficient using shRNA significantly sensitized these tumors to treatment and limited the emergence of drug resistance. Taken together, these data suggest that inhibition of REV1 or Polζ may have dual anti-cancer effects – sensitizing tumors to therapy and preventing the emergence of chemoresistance by limiting drug-induced mutagenesis.

Our findings suggest that this targeted approach would be applicable to oxaliplatin, satraplatin and picoplatin, in addition to cisplatin.

The chemosensitizing effects of targeting REV1 or Polζ is likely due to interference with TLS across intrastrand crosslinks and TLS associated with ICL repair. Disruption of REV1 or Polζ is associated with far greater sensitivities to cisplatin and other ICL-inducing agents when compared to disrupting other TLS polymerases, and these observations are consistent with both genetic and biochemical evidence implicating REV1 and Polζ in promoting ICL repair (Hicks et al., 2010; Niedzwiedz et al., 2004; Nojima et al., 2005; Raschle et al., 2008). Repair of ICLs requires a complex interplay between the TLS, Fanconi anemia, and the homologous recombination pathways (Deans and West, 2011). The data presented here support the concept that targeting REV1 or Polζ would also interfere with repair of the structurally diverse adducts created by all four platinating agents when present as an ICL as demonstrated by inefficient resolution of ICL-induced DSBs, in addition to limiting DNA replication through these distorting lesions on a single strand of DNA. It is important to note that following the appearance and resolution of ICL-induced DSBs is an indirect measure of actual ICL repair. The phenotypes we observed in Polζ-depleted cells are very similar to cells defective in HR repair (e.g. RAD51
knock-down cells) and cells deficient in ICL repair due to defects in the Fanconi Anemia pathway suggesting that ICL repair is impacted to a significant degree (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Rothfuss and Grompe, 2004).

Interestingly, recent biochemical studies have characterized Polχ as being inefficient at mediating TLS across artificial ‘unhooked’ crosslinks (Ho et al., 2011). This is unexpected based on the model for ICL repair and since the majority of genetic evidence suggests a prominent role for REV3 in protecting from ICL-induced cytotoxicity and genomic instability. Although REV1 and Polχ are believed to be essential for replicating across unhooked ICL in preparation for homologous recombination, we propose an alternative model where REV1 and Polχ participate downstream during homologous recombination repair (Sharma et al., 2012). Recent evidence implicate both REV1 and Polχ in promoting DSB repair and genomic stability (Schenten et al., 2009; Sharma et al., 2011; Wittschieben et al., 2006). It is also becoming increasingly clear that Polχ contributes to tumor suppression (Wittschieben et al., 2010). Before REV1 or Polχ can be considered as targets for adjuvant therapy with platinating agents, these additional roles will need to be better understood.
Authorship Contributions

Participated in research design: Sharma, Canman

Conducted experiments: Sharma, Shah, Joiner, Roberts, and Canman

Contributed new reagents: N/A

Performed data analysis: Sharma, Canman

Wrote or contributed writing of the manuscript: Sharma, Canman
References


Footnotes

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**Figure Legends**

**Figure 1:** (A) The different adducts created by cisplatin, oxaliplatin, satraplatin, and picoplatin are illustrated. (B) Dose response relationships between platinum analogs and growth inhibition. HeLa cells were treated with different doses of platinating agent for 1 hour, washed and then cultured for 4 days. The number of cells present were counted and normalized to the number of cells present when grown in the absence of drug. Results shown are the mean ± SEM of at least four independent experiments.

**Figure 2:** The TLS pathway limits the DNA damage response after treatment with platinating agents. A) HeLa cells were transfected overnight with the indicated siRNAs and allowed to recover for 24 hours. Cells were treated with platinum-containing drug for one hour using concentrations expected to inhibit cell proliferation by 35 – 40% (10 μM cisplatin, 32 μM oxaliplatin, 6 μM satraplatin and 64 μM picoplatin). Cells were fixed 24 hours after drug treatment and stained for γ-H2AX to measure activation of the DNA damage response. B) HeLa cells were transfected with the indicated siRNAs and harvested two days later to assess knockdown efficiencies. Whole cell lysates were subjected to SDS-PAGE and immunoblotted for endogenous RAD18, RAD51, Polη, or Topoisomerase 1 (loading control). Rad18 levels were reduced to 14.1 ± 10.7%; RAD51 levels were reduced to 36.8 ± 7.5%; and Polη levels were reduced to 18.0 ± 3.0% of control levels as determined by densitometry analysis (n=3, mean ± SD). C) 293T cells were cotransfected with GFP-tagged REV1 and the indicated siRNAs. Two days later, whole cell lysates were subjected to SDS-PAGE and immunoblotted for GFP or Topoisomerase 1. REV1-specific siRNA reduced GFP-REV1 protein levels to 22 ± 7% of control (n=3, mean ± SD). D) HeLa cells were transfected with control or REV3-specific
siRNA. Two days later, REV3L and GAPDH mRNA levels were determined by rtPCR using gene specific primers. Shown is a representative ethidium bromide stained agarose gel. REV3L mRNA levels were reduced by 35% ± 1.3% compared to control as determined by densitometry analysis (n=3, mean ± SD).

**Figure 3:** REV1 and Polζ are essential for tolerance of platinum adducts. HeLa cells were transfected with siRNA and treated with the indicated platinum-containing drugs for one hour as in Figure 2 A. Cells were fixed 24 hours later, fixed, stained for γ-H2AX and DNA content (propidium iodide), and then analyzed by flow cytometry (A and B). Dot plots depicting the level of γ-H2AX staining versus DNA content are shown in panel A and the corresponding histograms depicting the DNA content per event are shown in panel B. Each dot plot is labeled with the percentage of cells displaying enhanced γ-H2AX staining. Each histogram is labeled with the percentage of cells residing in S and G2/M at the time of fixation. Cells can progress through the cell cycle and avoid replication stalling more efficiently in the absence of Polη as compared to REV1 and Polζ following treatment with oxaliplatin, satraplatin, or picoplatin. Shown are representative dot plots and histograms from three independent experiments.

**Figure 4:** Platinating agents induce enhanced Chk1 and RPA phosphorylation in the absence of REV1 and Polζ. HeLa cells transfected with the indicated siRNAs were treated with 10 μM cisplatin, 32 μM oxaliplatin, 6 μM satraplatin and 64 μM picoplatin for 1 hour, and harvested 24 hours later. Whole cell lysates were immunoblotted for phospho-Ser345-Chk1 or total Chk1 protein (A) or phospho-RPA32 (S4/S8) or total RPA protein (B). Immunoblots showing GAPDH immunostaining demonstrate equal loading. Immunoblots are representative of two independent experiments.
**Figure 5:** Cancer cells lacking REV1 or REV3 are hypersensitive to cisplatin, oxaliplatin, satraplatin, and picoplatin. A) HeLa cells were mock transfected or transfected with control siRNA or siRNA targeting REV1, REV3 or Polη. Cells were then seeded at known densities cultured with or without two different doses of cisplatin, oxaliplatin, satraplatin or picoplatin until the untreated wells approached confluency (7-10 days). The relative surviving fraction was determined by measuring absorbance of solubilized crystal violet staining in each well normalized to the corresponding untreated well. Cells transfected with siRNA specific for RAD51 and treated with individual drugs are shown for comparison. Data represent the mean ± SEM from at least three independent experiments. Open lines indicate comparisons of the means among all columns. Bracketed lines indicate comparisons between two means: ns, not significant; **, \( P < 0.01 \); ***, \( P < 0.001 \); one-way ANOVA. B) Wild type, Polη-/-, Polξ-/-, and REV3-/- (clones 332 or 504) were cultured in the presence of three different doses of cisplatin, oxaliplatin, satraplatin or picoplatin for 48 hours. Viability was determined by measuring the percentage of cells excluding propidium iodide by flow cytometry. Data represent the mean ± SEM of at least three independent experiments.

**Figure 6:** RAD51 and Polζ are necessary for resolving platinating agent-induced DSBs. REV3- and Polη- depleted HeLa cells were treated with cisplatin (5 \( \mu M \)), oxaliplatin (15 \( \mu M \)), satraplatin (2 \( \mu M \)) and picoplatin (25 \( \mu M \)) for one hour, washed, and then fixed 24 and 48 hours later. Cells were stained for S1981P-ATM and 53BP1 as surrogate markers of DNA DSBs. Nuclear DNA was stained with DAPI. A) The percentage of cells exhibiting 10 or more colocalized foci containing both S1981P-ATM and 53BP1 was determined. Data represent the mean ± SEM from four independent experiments where >100 cells were counted in each experiment. B) The difference in percentage of cells displaying \( \geq 10 \) colocalized foci containing
both S1981P-ATM and 53BP1 between 24 and 48 hours is shown. C) Representative images of control-, REV3- and Polη- depleted cells treated with satraplatin are shown. The presence or absence of Polη had little impact on the efficiency of ICL-induced DSB resolution.
A

Cisplatin

Oxaliplatin

Satraplatin

Picoplatin

B

Figure 1
Figure 2

A. Immunofluorescence images showing the localization of γ-H2AX foci in cells treated with different agents and siRNA against Pol η, REV1, REV3, and RAD18. Images are labeled for DAPI (DNA) and γ-H2AX (histone H2AX phosphorylated at serine 139).

B. Western blot analysis for Pol η, RAD18, RAD51, and TOPO1 expression under various conditions: mock, no siRNA, siControl, siREV1, siREV3, and siRAD51.

C. Western blot analysis for GFP (REV1) expression under different conditions: mock, no siRNA, siControl, and siREV1.

D. Western blot analysis for REV3L and TOPO1 expression under various conditions: mock, siControl, and siREV3.
A

Control  Cisplatin  Oxaliplatin  Satraplatin  Picoplatin

siControl  8%  9%  7%  9%  10%
siPolη  8%  37%  6%  21%  16%
siREV1  12%  56%  15%  40%  31%
siREV3  13%  69%  47%  61%  51%
siRAD18  14%  45%  11%  35%  26%

FITC (γ-H2AX)

DNA content (propidium iodide)

% γ-H2AX positive cells

B

Control  Cisplatin  Oxaliplatin  Satraplatin  Picoplatin

siControl  26%  31%  28%  32%  33%
siPolη  37%  63%  57%  46%  48%
siREV1  41%  62%  59%  59%  58%
siREV3  40%  74%  64%  69%  68%
siRAD18  40%  70%  59%  60%  55%

% cells in S and G2/M

DNA content (propidium iodide)

Events

Figure 3
Figure 5

A

Surviving Fraction

Cisplatin (μM)

Oxaliplatin (μM)

Satraplatin (μM)

Picoplatin (μM)

B

% Viability

Cisplatin (nM)

Oxaliplatin (nM)

Satraplatin (nM)

Picoplatin (μM)

- WT
- Polη −/−
- Poli −/−
- REV3 −/− 332
- REV3 −/− 504